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A rapid and efficient assay for the characterization of substrates and inhibitors of nicotinamide *N*-methyltransferase

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ABSTRACT

Nicotinamide *N*-methyltransferase (NNMT) is one of the most abundant small molecule methyltransferases in the human body and is primarily responsible for the *N*-methylation of the nicotinamide (vitamin B3). Employing the cofactor *S*-adenosyl-L-methionine (AdoMet), NNMT transfers a methyl group to the pyridine nitrogen of nicotinamide to generate N-methyl nicotinamde (NMA). Interestingly, NNMT is also able to *N*-methylate a variety of other pyridine-containing small molecules suggesting a secondary role for the enzyme in the

detoxification of xenobiotics. A number of recent studies have also revealed links between NNMT overexpression and a variety of diseases including multiple cancers, Parkinson's disease, diabetes, and obesity. To facilitate further study of both the substrate scope and potential for inhibitor development, we here describe the development of a new NNMT activity assay. The assay makes use of ultra-high performance hydrophilic interaction chromatography (UHP-HILIC) allowing for rapid separation of the reaction products, coupled with quadrupole-time-of-flight mass spectrometric (QTOF-MS) detection, providing for high sensitivity and permitting the analysis of small samples. We successfully demonstrated the robustness of the method by performing kinetic analyses of NNMT-mediated methylation for a range of pyridine-based substrates. These findings also provide new insight into the diversity of substrate recognition by NNMT in a quantitative manner. In addition, we further established the applicability of the assay in the characterization of small molecule inhibitors of NNMT. To do so we investigated the inhibition of NNMT by the nonspecific methyltransferase inhibitors sinefungin and *S*-adenosyl-L-homocysteine revealing IC_{50} values in the low micromolar range. The results of these inhibition studies are particularly noteworthy as they will enable future efforts toward the development of new NNMT-specific inhibitors.

INTRODUCTION

The methyltransferases are a class of enzymes that methylate DNA, RNA, proteins, and small molecules using nature's general methyl donor *S*-adenosyl-L-methionine (AdoMet) as a cofactor. Protein methyltransferases have gained much attention in recent years given their roles in the post-translational modification of many epigenetic targets implicated in gene expression. By comparison, the non-protein methyltransferases (NPMTs) have generally received less attention.

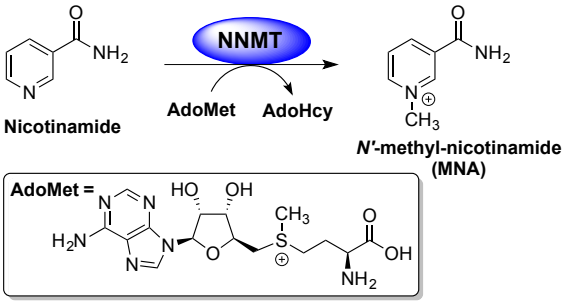


Figure 1. Methylation of nicotinamide by *S*-adenosyl-*L*-methionine (AdoMet) catalysed by nicotinamide *N*-methyltransferase (NNMT), forming *N'*-methyl-nicotinamide (MNA) and *S*-adenosyl-*L*-homocysteine (AdoHcy).

NPMTs represent group of methyltransferases that methylate various small molecules that are typically involved in metabolic pathways, signal transduction, and the biosynthesis or modification/detoxification of bioactive molecules.[1] One such NPMT is nicotinamide *N*-methyltransferase (NNMT, EC 2.1.1.1), which catalyses the *N*-methylation of nicotinamide (NA) using AdoMet to form *N'*-methylnicotinamide (MNA) and *S*-adenosyl-*L*-homocysteine (AdoHcy) as depicted in figure 1. In addition to nicotinamide, previous reports using NNMT isolated from porcine liver found that the enzyme is also able to methylate a range of other pyridine-containing compounds.[2] In the human body NNMT is expressed mainly in the liver, but has been found in lower levels in other organs as well.[3,4] NNMT's primary role in the liver has traditionally been viewed as serving various detoxification and metabolic pathways.[5] Recently, however, additional roles have also been attributed to NNMT indicating involvement in a range of disease states and physiological processes including Parkinson's disease, numerous cancers, metabolic disorders, obesity, and lifespan regulation.[6–12] The growing body of knowledge implicating NNMT in both healthy and disease states also suggests that the enzyme might be an interesting therapeutic target. Facilitating the design of new NNMT inhibitors is the recently

published crystal structure of NNMT which provides key insights in the active site residues and their interaction with the enzyme's substrates.[13] Also crucial to the development of inhibitors is a reliable and efficient assay method for quantifying NNMT's methylation activity towards nicotinamide and the various other substrates that have been identified and proposed to date.

A general method for assaying methyltransferase enzymes involves measuring production of the AdoMet by-product AdoHcy. This approach can however, be complicated by unpredictable background levels of AdoHcy commonly encountered in such assays. Such background AdoHcy can be the result of contamination in the AdoMet used, formation of AdoHcy via automethylation, or by the nonenzymatic decomposition of SAM into AdoHcy.[14,15] Other analytical methods more specific for measuring the activity of NNMT have also been developed. One approach is to use radiolabeled AdoMet which allows for quantification of NMNT activity by detecting the amount of radioactive MNA produced.[2,16] As an alternative, high performance liquid chromatography (HPLC) coupled with UV or fluorescence-based detection can also be used to assess the production of MNA by NNMT.[17,18] These methods however, are complicated by the use of complex buffer systems, tedious work-up, derivatization reactions, long run times, or the use of radioactive reagents.[2,16–18] Radiochemical or derivatization-based methods are not ideally suited for measuring large numbers of samples or for screening libraries of potential inhibitors. Also, while a recently reported HPLC-UV method for measuring NNMT activity in human liver cytosol allowed for the determination of kinetic parameters of NNMT,[18] the assay conditions used are also not optimal for screening purposes.

In developing an alternative approach to assaying the activity of NNMT we chose to employ a Hydrophilic Liquid Interaction Chromatography (HILIC) separation technique coupled with a mass spectrometric-based system detection system. As the product(s) of the enzymatic reaction

catalysed by NNMT are generally methylated pyridines, the analytes of interest will be inherently positively charged compounds. Such polar compounds can be analysed using Hydrophilic Liquid Interaction Chromatography (HILIC) without the need for derivatization or the application of ion-pairing agents. These factors also make HILIC highly compatible with selective and sensitive mass spectrometric (MS) detection.[19] In addition, the use of ultra-high-performance (UHP) column material in HILIC offers greater separation efficiency, sensitivity, and the admissibility of higher flow rates relative to conventional C_{18} HPLC-based separations leading to significantly shorter analysis times and higher sample throughput.

To demonstrate the applicability of our UHP-HILIC-MS method for the detection of methylated pyridines, a range of pyridine-containing compounds were tested as substrates for NNMT. A set of previously identified substrates, based upon the activity of NNMT isolated from porcine liver, formed the basis of our quantitative substrate recognition screen.[2] This substrate screen was also expanded to include other physiologically relevant pyridines so as to gain further insight into the NNMT substrate scope. The UHP-HILIC-MS method here described shows high sensitivity and selectivity for the detection of methylated pyridines. It further benefits from convenient sample work-up and very short run-times in the analysis of pyridine-containing compounds as substrates for NNMT. A subset of alternate NNMT substrates for which methylation activity was found to be similar to that of nicotinamide were identified and the kinetic parameters V_{max}, K_m and k_{cat} determined. Furthermore, we demonstrate that the assay can be readily adapted for use in screening inhibitors of NNMT as demonstrated by establishing the IC_{50} values for AdoHcy and the general methyltransferases inhibitor sinefungin.

EXPERIMENTAL SECTION

Cloning, Expression, and Purification of recombinant human NNMT

Total RNA was isolated from normal human renal tissue using the SV Total RNA Isolation Kit (Promega, Madison, WI, USA) as per previously described.[20] Total RNA (2μg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and random primers. cDNA was then subjected to PCR using the primers 5'-TCACATATGGAATCAGGCTTCA-3' (forward) and 5'-CTAAAGCTTTCACAGGGGTCTG-3' (reverse) to amplify the human NNMT open reading frame and to insert NdeI and HindIII restriction sites. The amplified and digested product was then cloned into a pET-28a plasmid vector to obtain the expression construct pET-28a-wt-hNNMT, which was subsequently transformed into E. Coli BL21 (DE3). Human recombinant NNMT was isolated from IPTG-induced cells and purified to homogeneity using nickel affinity purification as previously described.[13] Purity was confirmed using SDS-PAGE/Coomassie blue staining and NNMT identity was confirmed using SDS-PAGE/Western blotting.

Catalytic activity of recombinant protein was evaluated as previously reported, with minor modifications,[13] with 1 U of enzyme activity representing the formation of 1 nmol of MNA per hour of incubation at 37 °C. Homogeneous recombinant NNMT specific activity was 6740 U/mg protein and 6367 U/mg protein for the two batches of NNMT used with protein concentrations of 1.3 mg/mL and 0.94 mg/mL respectively.

Enzymatic Activity Assay

Enzyme activity assays were performed as previously described[13] with NNMT (16.25 μg/mL, 550 nM) in 50 mM Tris buffer (pH 8.6) containing 1 mM DTT (all final concentrations). The enzyme was incubated with the substrate pyridine compound for 5 minutes at 37°C using a heat

block before initiating the reaction by addition of AdoMet (for substrates that were not soluble in water, stock solutions were prepared in DMSO and diluted to a final concentration of <5% DMSO in the assay mixture. As a control, the methylation of nicotinamide was also measured in the presence of 5% DMSO revealing no difference in enzymatic activity. For these screens the final reagent concentrations were 100 μM AdoMet and 2.0 mM substrate and a series of time points were initially investigated (5, 15, 30, and 60 minutes). At each time point 15 μL aliquots of the reaction mixture were taken and added to 70 μL acetonitrile which served to quench the reaction by causing precipitation of the enzyme. Samples were then centrifuged for 5 minutes at 3,000 rpm and the supernatant analysed.

In cases where an increase in signal for the methylated product was observed, a second screen was performed in order to establish the initial velocity conditions of the particular substrate. For the more comprehensive screening, final reagent concentrations were 0.02, 0.05, 0.2, 0.5, 2.0, 5.0, 20 and 50 mM substrate and 40 μM AdoMet. The time-points used were 5, 15, 30 and 60 minutes after which 15 μL aliquots of the reaction mixture added to 70 μL acetonitrile (containing the relevant internal standard) and the sample worked-up as described above. For each of the substrates investigated in this screen the trideuteromethylated analogue was prepared for use as an internal standard. In addition, the non-isotope enriched methylated versions of each substrate were synthesized and used to confirm signal linearity. After establishing the suitable initial velocity conditions for each substrate the NNMT activity assay was performed in triplicate using a substrate concentration range of 0.02, 0.05, 0.2, 0.5, 2.0, 5.0, 20 and 50 mM and 40 μM AdoMet. The data thus generated was used in determining the kinetic parameters K_m, V_{max} and k_{cat} for each substrate investigated.

UHP-HILIC-MS method for analysis of methylated products

UHP-HILIC-MS analysis was performed using an 1290 Infinity UHPLC system (Agilent Technologies, Waldbronn, BW, Germany) consisting of a binary pump, an autosampler and a temperature controlled column department at 65°C with a Waters (Milford, MA, USA) acquity UPLC BEH amide 1.7 μM 3.0x100 mm HILIC column coupled to a Q-TOF II mass spectrometer with an electrospray ionization source and liquid chromatography sprayer from Bruker Daltonics (Bremen, HB, Germany), operated in positive mode. The compounds were eluted isocratically with 40% water containing 300 μM formic acid and 550 μM NH_4OH (pH 9.2) and 60% acetonitrile (% v/v) at a flow rate of 0.6 mL/min. The injection volume was 20 μL. MS settings were optimized for MNA signal-to-noise ratio. The optimal settings were a capillary voltage of 1500 V, a nebulizer pressure of 4.1 bar, a drying gas flow rate of 9.8 L/min, a drying temperature of 220 °C, a scan range of *m/z* 90-400 and a spectra sample rate of 3 spectra/s.

Analytical Method Validation

The MNA analysis was validated between 0.31-100 μM for within and between run accuracy and precision, linearity of calibration curve, sample recovery and limit of detection. For the other substrates a system suitability test was performed, consisting of a linearity test and within run accuracy and precision test at the same concentration levels as for MNA.

Linearity was performed with calibration points consisting of 0.31, 0.62, 1.25, 2.5, 5, 10, 25, 50 and 100 μM MNA in water. Quality control (QC) samples consisted of 50 mM Tris Buffer, 1 mM DTT and 16.25 μg/mL (550 nM) NNMT, and MNA concentrations of 0.31, 10 and 100 μM (all final concentrations). Samples for analysis were worked-up as described above in the enzymatic activity assay section and analysed with the optimized UHP-HILIC-MS method. Ratios of MNA and internal standard d_3-MNA were calculated and plotted against the

concentration. Linearity was assessed visually and by calculation of Pearson's *r*, which should be >0.95. Sample recovery was calculated by analysing a treated QC sample with UHP-HILIC-MS and by comparing this signal with the signal obtained by the analysis of an untreated sample in acetonitrile:water (80:20%, v/v) with the same concentration. The sample recovery was 77±2% and 83±4% for MNA and d_3-MNA, respectively (n=8). All accuracy and precision results were within the pre-set limits of 85-115% for accuracy and <15% for precision (Table 1).

The accuracy was tested by analysing the QC-samples using the complete method, including sample pre-treatment and by calculating the percentage of determined concentration with respect to the nominal value. The precision was defined as the coefficient of variation of the accuracy results. Accuracy and precision tests were performed in six-fold per concentration in 1 run and in one-fold per concentration in 3 separate runs. The acceptance criteria of the accuracy results were 85-115% and of the precision results <15%. The limit of detection was obtained by analysis of 8 pre-treated samples corresponding to a signal-to-noise ratio of 3 (Table 1). All other methylated substrates were analyzed with the same analytical method and a system suitability

Table 1. Validation parameters of the UHP-HILIC-MS method for MNA

QC conc. (µM)	Pearson's r	
0.31 - 100	0.996 – 0.998	
	Accuracy (%)	Precision CV (%)
Within run (n=6)		
0.31	87.5	2.9
10	100.5	3.6
100	86.9	2.1
Between runs (n=3)		
0.31	85.3	4.7
10	104.8	3.6
100	88.0	0.8
Limit of detection (S/N ≥ 3)	0.16 µM	

test performed prior to analysis. This test involved confirming calibration curve linearity as described for MNA and an accuracy determination by analysis of QC samples at concentrations of 0.31, 10, and 100 μM. The calibration curves were found to be linear from 0.31-100 μM and within-run accuracies and precisions were within the 85-115% range and <15%, respectively.

Data Analysis

The data obtained from the UHP-HILIC-MS system included a linearity line with ten different concentrations of reference standard (from 0.16-100 μM) and a fixed concentration of internal standard (10 μM). These data-points were subjected to weighted regression ($1/x^2$). The intercept and slope were used for determination of the measured concentrations.

For quantification of the methylated product, the ratio of analyte to internal standard was calculated by integrating the corresponding peak areas in the chromatogram of test samples. The ratio was quantified using the linearity line obtained with the reference standards. The concentrations were then converted to enzyme velocity in nmoles produced/hour/mg hNNMT protein using equation 1 with the concentration of the methylated product in nM, time in minutes, and the enzyme concentration in mg/L.

$$v = \frac{[product]*\frac{60}{t}}{[E]} \qquad \text{Equation [1]}$$

Calculation of V_{max} and K_M was done using Graphpad Prism 6 following non-linear (Michaelis-Menten) regression analyses using equation 2.

$$v = \frac{Vmax.[S]}{Km+[S]} \qquad \text{Equation [2]}$$

The k_{cat} was calculated from the V_{max} using equation 3 with V_{max} in nmol/hour/mg enzyme and enzyme concentration in mg/L. To obtain k_{cat} with the unit s^{-1}, the maximum velocity V_{max} is divided by 3600.

$$k_{cat} = \frac{V_{max}}{[E]} \qquad \text{Equation [3]}$$

In cases where the product formation was fund to decrease at high concentration of substrate, the data was fitted to an expression accounting for substrate inhibition (equation 4).

$$v = V_{max} * \frac{[S]}{Km+[S]*\left(1+\frac{[S]}{Ki}\right)} \qquad \text{Equation [4]}$$

Inhibition assays

The NNMT inhibitory activity of the general methyltransferase inhbitors AdoHcy and sinefungin was evaluated using the UHP-HILIC-MS method. In this assay the substrate concentrations used were set at values near their measured K_m values (10 μM AdoMet and 200 μM nicotinamide). AdoHcy and sinefungin were applied at ten different concentrations between 10 nM and 5000 μM, measured in duplicate. The inhibitors were first incubated with NNMT for 10 minutes at 37°C, after which the reaction was initiated by addition of a mixture of nicotinamide and AdoMet. After a reaction time of 10 minutes at 37°C a 15 μL aliquot was taken and added to 70 μL of acetonitrile (containing 10 μM d_3-MNA as internal standard). Quantification of the MNA generated was achieved as described above. Blank samples (no enzyme) and positive controls (no inhibitor) were also performed. The percent activity values were plotted as a function of inhibitor concentration and fitted using non-linear regression analysis of the Sigmoidal dose–response curve generated using normalized data and a variable slope following equation 5,

$$Y = \frac{100}{(1+10^{((LogIC50-X)*HillSlope))})} \qquad \text{[Equation [5]}$$

where Y = percent activity, X = the logarithmic concentration of the compound and Hill Slope = slope factor or Hill coefficient. The IC_{50} value was determined by the concentration resulting in a half-maximal percent activity. The standard errors of the mean (S.E.M.) were reported using the symmetrical CI function in Graphpad Prism 6.

RESULTS AND DISCUSSION

Analytical Method Development

To achieve a rapid and efficient method for measuring the methylating activity of NNMT we employed an UHP-HILIC-MS approach to detect MNA and other methylated pyridine products of the enzymatic reaction. The method developed was optimized so as to achieve maximum detection sensitivity and sample throughput. In optimizing the UHP-HILIC aspect of the method we examined the influence of the mobile phase composition and pH, column temperature, and flow rate on the elution profile of a standard MNA sample. Initial attempts employed isocratic elution with a mobile phase consisting of 40% acetonitrile containing 50 μM formic acid (pH 2.2) at a flow rate of 0.5 mL/min at 25°C. This method yielded broad and tailing chromatographic peaks for MNA. Increasing the amount of formic acid (up to 300 μM) improved peak shape and decreased retention time. As MNA is inherently positively charged, an acidic eluent is not essential to obtain sufficient MS response so we also investigated the use of alkaline mobile phases. An increase in eluent pH by addition of NH_4OH decreased the background noise without affecting the peak area for MNA and considerably improved efficiency while further shortening retention time due to the higher salt concentration. Optimal

peak shape and retention time were obtained with an eluent containing 300 μM formic acid and 550 μM NH_4OH (pH 9.2). While these are relatively high concentrations of eluent additives for use in MS, we found that they did not lead to MNA signal suppression relative to the initial analyses performed using eluent containing 50 μM formic acid. An increase in column temperature (up to 65°C) resulted in a lower back pressure caused by a lower solvent viscosity and an enhancement of efficiency due to an increase in mass transfer. The effect of flow rate was also studied within a range of 0.2-1.0 mL/min. As expected, the highest flow rate tested decreased retention time but had the additional effect of narrowing the MNA peak at baseline to just 4.5 seconds making it difficult to acquire enough data points to define peak shape. The amount of data points could be increased by raising the spectra acquisition rate of the MS instrument but this also negatively affected MS response. As a compromise, a flow rate of 0.6 mL/min and an acquisition rate of 3 spectra/s were chosen to ensure at least 10 data points to define the peak and to obtain an acceptable retention time and MS response. With these optimized parameters a chromatogram of an MNA sample could be acquired every 2.0 min while assuring optimal chromatographic conditions (Figure 2). The analysis of other pyridine-based

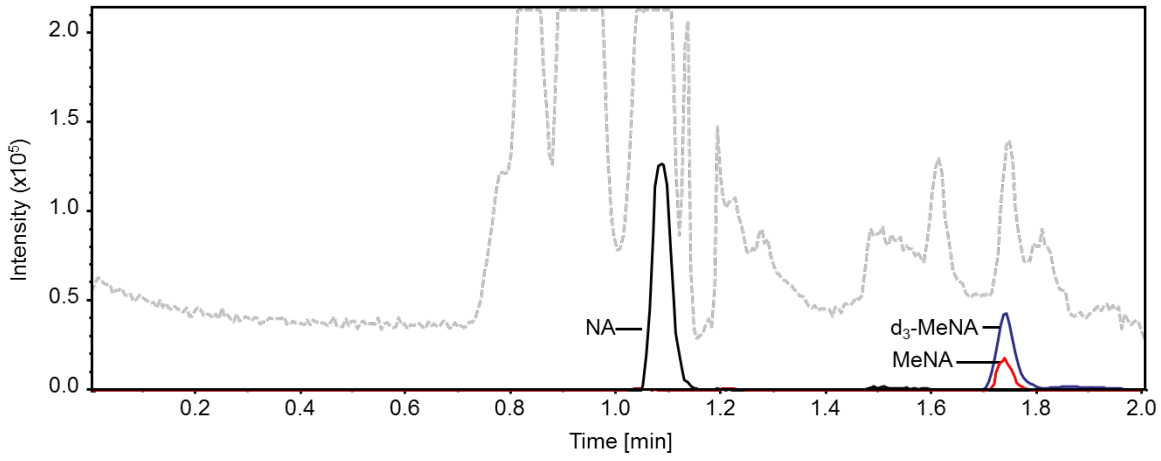
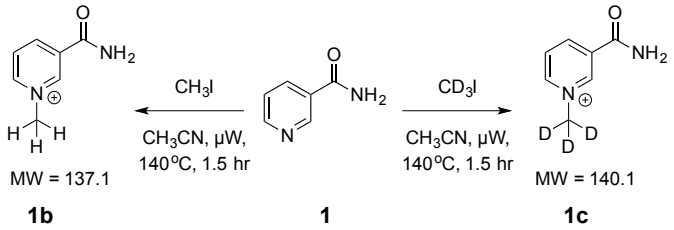


Figure 2. Representative UHP-HILIC-MS trace for the NNMT-catalysed methylation of nicotinamide. Indicated are: the total ion chromatogram of the assay mixture (grey dashed line) and the extracted ion chromatograms for nicotinamide (NA, *m/z* 123.1, black line), *N*-methylnicotinamide (MNA, *m/z* 137.1, red line) and *N*-trideuteromethylnicotinamde (d3-MNA, *m/z* 140.1, blue line).

NNMT substrates was also successfully performed using the same approach showing the method to be generally applicable.

Internal Standards

In order to quantify formation of the methylated pyridine products for nicotinamide and all other pyridine substrates, trideuterated versions were employed as internal standards. The trideuterated species have the same retention time and behaviour as their non-deuterated versions but can be separated based on *m/z* difference and can thereby be used to correct for matrix effect, signal suppression, and other effects due to sample preparation. A rapid and reliable procedure for the synthesis of both the non-deuterated reference standards and the trideuterated internal standards was developed (Scheme 1). Reaction of the pyridine compounds with either CH_3I or CD_3I under microwave irradiation followed by a simple workup consisting of filtration and washing of precipitated product generally provided the methylated reference standards as their iodide salts in high purity. For two of the substituted pyridines identified as alternate NNMT substrates (thionicotinamide **5** and 2-methoxypyridine **10)** this general procedure was not suitable and alternative syntheses of the methylated products were employed (see supporting information).



Scheme 1. General procedure used for the synthesis of reference standards (using methyl iodide), and internal standards (using trideuteromethyl iodide).

Analysis of NNMT activity and substrate tolerance

We next examined the suitability of the UHP-HILIC-MS method in determining the kinetic parameters for the various NNMT substrates. To begin we first established the K_m values for AdoMet and nicotinamide. For the K_m determination of nicotinamide a fixed concentration of 40 μM AdoMet was used with a range of nicotinamide concentrations from 0.02-50 mM. For the AdoMet K_m determination a fixed concentration of 2 mM nicotinamide was employed with AdoMet concentrations ranging from 0.2-200 μM. Using this approach the K_m value determined for nicotinamide was 199 μM and for AdoMet 8.5 μM (Figure 3). Both K_m values are in good agreement with those previously reported by others using different assay methods for studying NNMT activity.[4,21]

As described in the experimental section above, initial screens were also performed with a number of other pyridine-containing compounds (Figure 4) to identify alternative NNMT substrates. In cases where the preliminary screen revealed significant methylation activity (compounds **1**-**11**), the initial velocity conditions for each substrate were established and the kinetic parameters K_m, V_{max} and k_{cat} determined. Direct comparison of all substrates was done

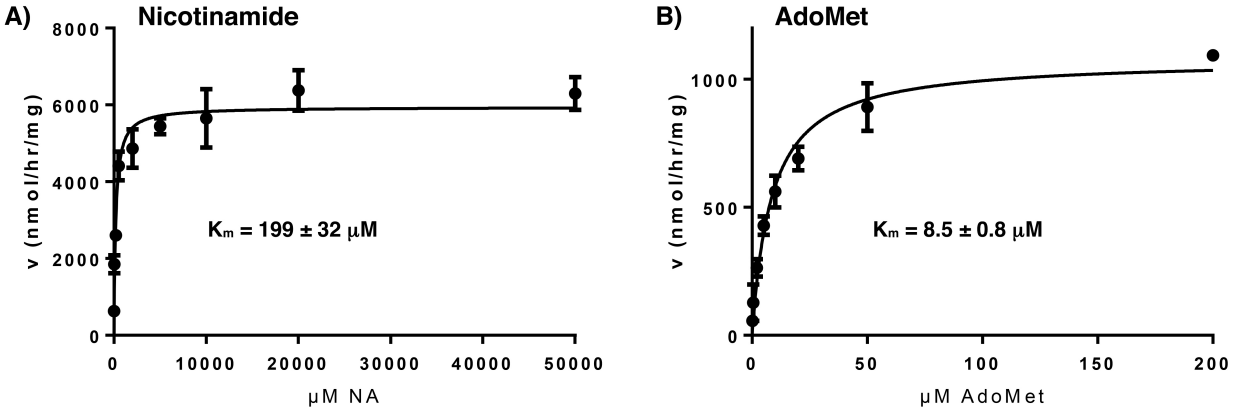


Figure 3. Michaelis-Menten curves for determination of the kinetic parameters of NNMT substrates nicotinamide and AdoMet. **A)** Concentration of nicotinamide varied (AdoMet 40μM fixed concentration) and **B)** Concentration of AdoMet varied (nicotinamde 2 mM fixed concentration).

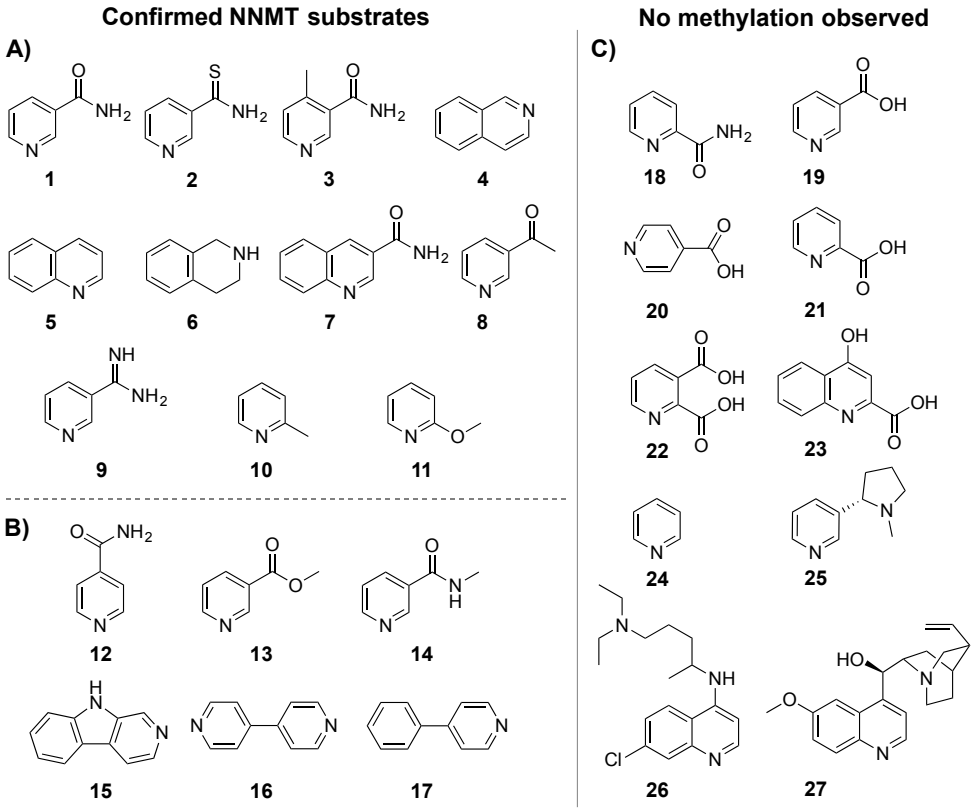


Figure 4. Substrates analysed for methylation by NNMT: **A)** Compounds **1-11** were confirmed as NNMT substrates with significant methylation activity. **B)** Compounds **12-17** were also found to be methylated but at a much lower extent. **C)** Compounds **18-27** showed no detectible methylation.

based on the k_{cat}/K_m ratio of each substrate, a measure for the catalytic efficiency of an enzyme.[22] The kinetic parameters summarized in table 2 show that compounds **2** and **3** behave as effective NNMT substrates with catalytic efficiencies comparable to nicotinamide while those of compounds **6**-**11** are significantly lower. While compounds **12**-**17** were also confirmed as NNMT substrates, their much lower rates of methylation made it difficult to establish initial velocity conditions using our method and K_m, V_{max} and k_{cat} values were therefore not determined.

The data clearly demonstrates that the position of the amide moiety on the nicotinamide ring is crucial for activity as only minor methylation was observed for isonicotinamide **12** (less than 2% relative to nicotinamide) and no methylation could be detected for picolinamide **18**. Also, when the amide moiety is replaced with a carboxylic acid, the activity disappears completely as

Table 2. Kinetic parameters of confirmed NNMT substrates ranked according to their relative catalytic efficiency

#	Substrate	K_m (mM)	V_{max} (nmol/hour/mg)	k_{cat} (s-1)	k_{cat} / K_m (s-1 mM-1)	Relative k_{cat} / K_M
1	Nicotinamide	0.199 ± 0.032	5942 ± 154	0.10157	0.5104	1.000
2	Thionicotinamide	0.111 ± 0.042 (K_i 6.6 ± 2.4)	1411 ± 181	0.03336	0.3005	0.589
3	4-Methylnicotinamide	0.335 ± 0.058	5344 ± 158	0.09135	0.2727	0.534
4	Isoquinoline	0.087 ± 0.025 (K_i 20.2 ± 5.4)	1005 ± 75	0.01718	0.1975	0.387
5	Quinoline	0.609 ± 0.197 (K_i 10.2 ± 3.3)	2713 ± 398	0.04639	0.0762	0.149
6	Tetrahydroisoquinoline	0.613 ± 0.148 (K_i 4.7 ± 1.2)	1915 ± 254	0.04527	0.0739	0.145
7	Quinoline-3-carboxamide	0.364 ± 0.123 (K_i 9.7 ± 3.5)	310 ± 44	0.00733	0.0201	0.039
8	3-Acetylpyridine	0.727 ± 0.115	845 ± 25	0.01444	0.0199	0.039
9	Nicotinimidamide	1.326 ± 0.280	790 ± 34	0.01350	0.0102	0.020
10	2-Methylpyridine	2.029 ± 0.239	608 ± 16	0.01039	0.0051	0.010
11	2-Methoxypyridine	0.548 ± 0.213	155 ± 11	0.00265	0.0048	0.010

seen for compounds **19-21**. Methylation was also not observed for quinolinic acid **22** and kynurenic acid **23**. These findings can be explained by the presence of an aspartic acid residue in the active site (D197) that coordinates with the amide moiety of nicotinamide.[13] It is likely that replacing the amide unit with a carboxylic acid group results in a repulsive interaction. A significant decrease in methylation was also observed for the nicotinamide analogues bearing a methyl ester (**13**) or *N*'-methylamide (**14**) replacement at position 3, again emphasizing the importance of the amide moiety in substrate recognition. Nicotinamide analogue bearing a thioamide (**2**), acetyl (**8**), or amidine (**9**) group at the 3-position were well tolerated. Simple pyridines containing either methyl (**10**) or methoxy (**11**) groups at the 2-position were also methylated albeit at a somewhat lower rate. Interestingly, no evidence of methylation was detected for pyridine (**24**) itself, tested as both the free-base and tosylate salt.

In agreement with the findings of Alston and Abeles who studied the substrate scope of NNMT isolated from porcine liver,[2] quinolines **4**-**7** were also found to be effective NNMT

substrates. Interestingly, methylation was also observed for the non-pyridine substrate 1,2,3,4-tetrahydro-isoquinoline **6**. These observations suggest that additional aromatic rings can be accommodated in the active site of the enzyme. The position of additional aromatic rings relative to the site of methylation also appears to be important as relatively weak methylation is observed for norharmane **15**, bipyridine **16** and 4-phenylpyridine **17**. Of particular note is a long-held interest in the metabolism of **17** given that its methylated product is a putative neurotoxin that has been implicated in causing Parkinson's disease phenotypes.[23] While the NNMT-mediated methylation of **17** has been proposed in the literature,[23] our data is the first to show that it can in fact be methylated by NNMT *in vitro*. Unique among the different compounds evaluated as NNMT substrates are tetrahydroisoquinoline **6** and bipyridine **16** as both could, in principle, undergo two methylations. The data obtained for the incubation of these compounds with NNMT however, provided no signal indicative of the doubly methylated products. In addition to the relatively simple compounds discussed above, more complex pyridine compounds of biological relevance were also evaluated as NNMT substrates. To this end nicotine **24,** chloroquine **26**, and quinine **27** were tested but showed no methylation activity, presumably due to an inability to access the NNMT active site.

Of note are the substrate inhibition kinetics observed for thionicotinamide **2** and quinolines **4**-**7**. Compounds **2** (K_i = 6.6 mM), **4** (K_i of 20.2 mM), **5** (K_i of 10.2 mM) **6** (K_i of 4.7 mM), and **7** (K_i of 9.7 mM) all inhibit the enzyme when administered at high concentrations (see supplemental information section for inhibition curves). This data may point towards the presence of an allosteric binding site through which the activity of NNMT might be regulated. While the concentration of these compounds needed to cause inhibition may not be

physiologically relevant, the results provide new insight into the possibility of inhibiting NNMT via interactions at alternate binding sites.

Inhibition studies

Among the various methods developed to date for studying NNMT activity, none have yet been applied towards characterizing NNMT inhibition. We therefore next evaluated the application of the UHP-HILIC-MS based method for assessing the inhibition of NNMT by two general and well-studied methyltransferase inhibitors, AdoHcy and sinefungin. Given their structural similarity with AdoMet these compounds are capable of inhibiting a wide range of AdoMet-dependent methyltransferases (Figure 5).

The UHP-HILIC-MS assay conditions were modified to achieve optimal results for inhibition screening rather than activity screening. For the assay used in studying NNMT activity with various substrates the cofactor AdoMet was employed at saturating conditions. For studying NNMT inhibitors that are competitive for AdoMet and/or nicotinamide binding, however, it is important that both substrates be used at concentrations near their K_m values so as to achieve reliable inhibition data.[24] The concentrations of AdoMet and nicotinamide were therefore set at 10 μM and 200 μM respectively. For the purposes of generating IC_{50} curves for AdoHcy and sinefungin the inhibitors were incubated with NNMT over a concentration range of 10 nM to

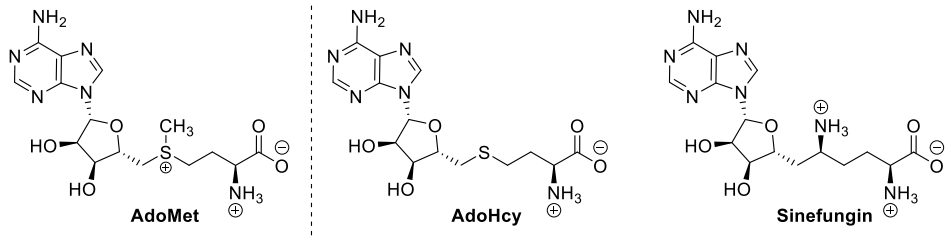


Figure 5. The structures of cofactor AdoMet and general methyltransferase inhibitors AdoHcy and sinefungin

5000 μM. The enzyme reaction was initiated by addition of nicotinamide/AdoMet mixture and left to react for 10 minutes prior to sampling for analysis. Based upon the residual NNMT activity measured, inhibition curves were generated giving IC_{50} values of 26.3 ± 4.4 mM and 3.9 ± 0.3 mM for AdoHcy and sinefungin respectively (Figure 6). These findings demonstrate the utility of the UHP-HILIC-MS assay here developed for obtaining detailed NNMT inhibition data.

CONCLUSION

We here describe the development of a rapid and convenient analytical method for measuring the activity of NNMT based on the use of an UHP-HILIC-MS approach. The method can be reliably used for the detection and quantification of NMA as well as a range of other positively charged, methylated pyridines. This assay represents a significant improvement over existing methods used for studying the activity of NNMT and specifically benefits from run-times of a little as two minutes, convenient sample preparation, high sensitivity, and the use of readily synthesized deuterated internal standards.

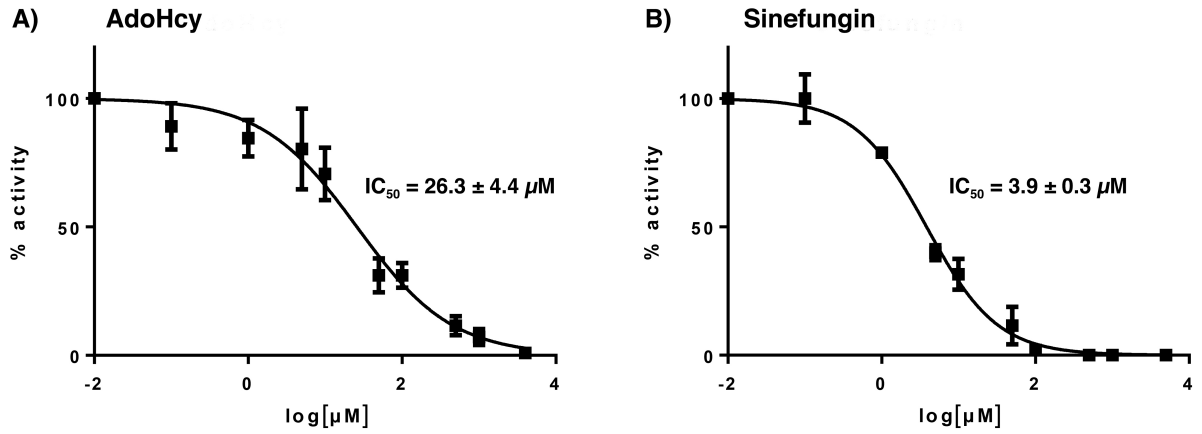


Figure 6. IC_{50} curves for **A)** AdoHcy and **B)** Sinefungin based on 10 different concentrations measured in duplicate

The broad applicability of the method was also demonstrated by studying the NNMT-mediated methylation of a wide range of substituted pyridines. In doing so, the kinetic parameters K_m, V_{max} and k_{cat} where determined for a number of alternate NNMT substrates. The results provide insight into the methylating abilities of NNMT *in vitro* along with detailed information on the relative catalytic efficiency for each substrate. From the data obtained it is clear that NNMT can accommodate a relatively wide variety of pyridine-containing small molecules. Whether methylation of such alternate NNMT substrates is relevant *in vivo* is unclear given that they would also have to compete with nicotinamide. However, given that cellular nicotinamide levels can fluctuate significantly (depending, for example, on dietary intake) the likelihood of alternate substrates being methylated by NNMT may also vary accordingly.

We here also demonstrated the applicability of the UHP-HILIC-MS method for assessing the inhibitory effects of small-molecule NNMT inhibitors. To date, none of the other NNMT assays developed have been shown to be applicable in providing detailed inhibition data. We found our method to be readily adaptable in generating NNMT inhibition curves and IC_{50} values for the known methyltransferase inhibitors AdoHcy and sinefungin. Given the increased interest in NNMT as a therapeutic target it is likely that the method here described will be of great value in identifying new NNMT inhibitors. Importantly, the rapid and convenient nature of the assay should also lend itself to the screening of inhibitor libraries. In this regard investigations are currently underway in our group aimed at developing novel NNMT-selective inhibitors, the results of which will be presented in due course.

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and analytical data for all new compounds including ^{1}H and ^{13}C NMR spectra, HRMS, and supporting figures for Michaelis-Menten and substrate inhibition kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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